Use of Tocopherol Extract and Different Nitrite Sources and Starter Cultures in the Production of Organic Botifarra Catalana, a Cooked Cured Sausage

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ABSTRACT

This research evaluates the effects of adding a tocopherol mix (200 mg/kg), two nitrite sources (sodium nitrite or a nitrate-rich vegetable concentrate) and the use of *Staphylococcus carnosus* together with three fermentation types that varied in temperature (12 h at 4 ºC or 16 ºC) on different quality parameters and acceptability of cooked cured sausages after vacuum packing and storage at 4 ºC for 120 days. In the presence of *S. carnosus*, residual nitrate and nitrite levels were reduced. Sausages containing vegetable concentrates and without *S. carnosus* resulted in higher amounts of residual nitrate and lower curing efficiency. The lowest values in redness and acceptability were observed in those sausages without starter cultures. The addition of tocopherols had no effect on oxidative status and susceptibility to oxidation. However, the highest amount of hydroperoxides was related with nitrite decreased formation. Overall, vegetable concentrates can be used as curing agents if fermentation with a nitrate-reducing starter culture is allowed.

KEYWORDS: cooked cured meat; organic meat products; nitrate and nitrite reduction; nitrate-reducing starter culture; vegetable concentrate, oxidation

1. INTRODUCTION

Fermented meat products have their roots in an age-old tradition and are mainly produced in the Western world (Hammes, 2012). Several types of cured meat products are produced in Spain, some of which are specialties from different regions. In Catalonia (Northeastern Spain), *botifarra catalana,* a traditional cooked cured sausage, is produced using a manufacturing process similar to that of cooked cured ham. The only curing agent permitted in the production of *botifarra catalana* is nitrite (European Commission, 2006), which provides the distinctive color of cooked cured meat. Nitrite also acts as an antimicrobial against *Clostridium botulinum*, prevents oxidation, and gives the sausage its cured flavor (Pegg and Shahidi, 2000). Despite all of these desirable effects, under certain conditions, nitrite can react with amines and amino acids in meat and produce *N*-nitrosamines, which play a role in human carcinogenesis. Therefore, the amount of curing agent that can be added to or contained in the cured product is regulated in Europe (European Commission, 2006) and the US (Sebranek and Bacus, 2007).

Consumer's interest in eco-labeled foods continues to grow because they perceive these products to be healthier, tastier and of higher quality, produced with animal welfare in mind and free of additives. In this context, the production of cured organic meat products involves the reduction and/or omission of curing agents (Sebranek and Bacus, 2007; European Commission, 2008). However, the application of this reduction and/or

omission without sufficient technological knowledge and modification of processes may result in poor sensory and microbiological quality (Hammes, 2012). As an alternative, vegetables and vegetable concentrates (VC), which have naturally occurring nitrates, have been used to circumvent the addition of curing salts to prevent the loss of the typical sensory characteristics of classical curing. Once added to sausages, nitrate needs to be reduced to nitrite to maximize the potential for introducing natural sources of nitrite into the processed meat (Sebranek and Bacus, 2007).

The introduction of starter cultures allowed the meat industry to control the fermentation process to ensure high standards of sensory quality and hygiene while reducing production times and costs. Some starter cultures also ensure that the added nitrate or nitrite is reduced to safe low limits (Hammes, 2012). The most efficient nitrate-reducing organisms are staphylococci and micrococci and these are therefore crucial for meats cured using nitrate sources (Sebranek and Bacus, 2007). Regarding the production of *catalana* sausages, in a previous study (Magrinya et al., 2012), a combination of two starter cultures (one containing lactic acid bacteria and the other containing *Staphylococcus carnosus* with intense nitrate reductase activity) was used and the fermentation time (at 16 ºC) required for nitrate reduction was optimized. However, considering that similar results were obtained for the different fermentation times assayed (6, 12 or 24 h), it was hypothesized that the progressive cooking used for the *catalana* sausages, which includes an initial step at 40 °C for 2 h, was responsible

for the almost complete nitrate reduction. Therefore, it is unclear if the fermentation with these starter cultures at 16 ºC offers a clear advantage compared to other alternative production procedures, resembling more the traditional process, in which the fermentation is carried at lower temperatures without the addition of starter cultures.

Lipid oxidation promotes rancidity changes and has also been correlated with meat discoloration (Wood et al., 2008; Parra et al., 2010). Nitrite exerts a relevant antioxidative effect acting by different mechanisms (Pegg and Shahidi, 2000). Therefore, reducing nitrite levels may increase the susceptibility of sausages to oxidation, thereby making it necessary to protect these meat products with antioxidants. Dietary supplementation with tocopherol acetate resulted in the stabilization of the desired color and inhibits lipid oxidation in cooked hams (Dineen et al., 2000). In a previous work, it was found that the addition of a tocopherol extract prevented from oxidation and improved red color of dry-fermented sausages during storage (Magrinya et al., 2009). *Botifarra catalana* is commonly distributed in retail markets vacuum-packed and under refrigeration conditions. In this regard, the addition of tocopherol in the formulation of this sausage may prevent oxidation during cooking and/or extend its shelf-life.

Therefore, the objective of the present study was to examine the effects of producing organic *botifarra catalana* using various methods, including the addition of tocopherol extract and VC to the sausage formula, and the fermentation at different conditions (12

h at 16 ºC *vs* 12 h at 4ºC) with and without the addition of different starter cultures, on various quality parameters.

2. MATERIAL AND METHODS

2.1. Reagents, standards and ingredients

Tocopherol extract (Guardian™ Toco 50, 50% mixed tocopherols) was obtained from Danisco (Copenhagen, Denmark). A bioprotective starter culture containing *Lactobacillus sakei* and *Staphylococcus xylosus* (B-FM SafePro™), a culture with intense nitrate reductase activity that contains *Staphylococcus carnosus* (CS-300 BactoFerm®) and a VC (Natasy CC 227) were obtained from CHR Hansen (Hørsholm, Denmark). Sodium nitrite, used as a pure sodium-nitrite source (99.6%), was obtained from Merck (Darmstadt, Germany). Sodium ascorbate, dextrose and salt were obtained from Espècies Teixidor (Manresa, Spain). Organic lean back meat and jowl fat were obtained from Embotits Salgot (Aiguafreda, Spain). Ground white pepper was obtained from Gewürzmüller (Korntal-Münchingen, Germany). Tocopherol standards were obtained from Calbiochem (San Diego, CA, USA). All chemicals used were of ACS grade except the solvents used in the induced ferrous oxidation–xylenol orange (FOX) method, the Hornsey method and the tocopherol plus tocotrienol determination, which were of HPLC grade.

2.2. Experimental design

Twelve treatments resulted from a 2x3x2 factorial design (Table 1) aimed at studying the effects of adding a tocopherol extract (0 and 200 mg of mixed tocopherols/kg of raw mixture), of three different types of fermentation (12 h at 16 ºC with bioprotective and nitrate-reducing cultures, type A; at 4 ºC with only a nitrate-reducing culture, type B; or, at 4 ºC without starter cultures, type C), and of two different sources of nitrite (pure sodium nitrite or a VC, each providing the equivalent of 70 mg of $NaNO_2$/kg raw mixture), on several quality parameters of cooked cured meat. The concentration of nitrite added was below the maximum level of ingoing sodium nitrite allowed in organic meat products in Europe (European Commission, 2008). A previous study that examined different fermentation times at 16 ºC demonstrated that type A yielded *botifarra catalana* with acceptable quality parameters (Magrinya et al., 2012). Type B was included because the nitrate-reducing culture used is, according to the producer, effective at low temperatures (10 ºC), and the progressive cooking procedure, including an initial step at 40 ºC for 2 h, favors its activity (Magrinya et al., 2012). Sausages produced in the different treatments were cooked and thereafter stored at 4 ºC for 0, 60 or 120 days. The inclusion of storage time as a factor thus resulted in 36 different samples.

2.3. Sausage preparation and sampling

With the exception of some of the factors studied, the sausage formulation and elaboration procedures used in this study are typical of *botifarra catalana*. A mixture

consisting of 40.8 kg of lean back meat plus 4.8 kg of jowl fat from organic pigs (both bought directly from Embotits Salgot) was used to prepare the ground meat. After homogenization, raw mixture was divided into two batches of 22.8 kg. For microbiological quality control of the meat mixture, 50 g of each batch was taken aseptically and analyzed as described below. Afterwards, 100 mL of sunflower oil with or without tocopherol extract was added to each batch. In each batch, the common ingredients (432 g salt, 72 g white pepper and 72 g dextrose) were added following dispersal in 300 mL of cold spring water. Following the addition of these ingredients, each batch was mixed for 90 seconds. To characterize these initial raw mixtures (moisture, crude fat, fatty acid composition, pH, and tocopherol and tocotrienol content), samples from these two mixtures, with and without tocopherol extract, were finely ground (Retsch knife mill, model Grindomix GM200; Haan, Germany) and vacuum packed in high-barrier multilayer bags (Cryovac® BB325; 130 x 180 mm; permeability to oxygen, 25 $cm^3 \cdot m^{-2} \cdot day^{-1} \cdot bar^{-1}$ at 23 ºC and 0% RH; approximately 20 g of meat/bag) and stored at -25 ºC until analysis.

Each batch was further divided in three, resulting in six different batches of 7.8 kg of raw mixture. The starter cultures, 0.25 g/kg of *S. carnosus* and 0.25 g/kg of the bioprotective culture, were added to the raw mixtures dispersed in 28 mL of cold spring water, in accordance with the experimental design (Table 1). The same amount of spring water was added to the raw mixtures without starter cultures. After this addition,

each batch was manually mixed for 5 minutes. The six batches were then divided in two, resulting in 12 batches of 3.88 kg, and samples of each batch were taken aseptically for microbiological analysis. The meat was then stored at 4 ± 2 ºC until the following day.

After storage, the nitrite source (50 mL of a dilution of pure $NaNO_2$ (5.66 mg $NaNO_2$/mL) or VC (0.26 g vegetable concentrate/mL) was added to the batches in accordance with the experimental design (Table 1). Ascorbic acid (0.5 g/kg) was added together with the nitrite sources. Subsequently, the raw mixtures were manually mixed for 2 minutes and stuffed into natural casings (50–55 mm in diameter). To check nitrite dose, samples from these 12 mixtures were finely ground (Retsch knife mill), vacuum packed in high-barrier multilayer bags (Cryovac® BB325; approximately 20 g meat/bag) and stored at -25 ºC until nitrate and nitrite analysis. The pH of the 12 raw mixtures was also determined. Five sausages per treatment weighing around 500 g were fermented for 12 h at 16 ºC or 4 ºC in accordance with the experimental design (Table 1). After this period, samples were taken aseptically for microbiological analysis and the pH was measured again. Sausages were then vacuum packed (Cryovac® HT3050; 325 x 550 mm; permeability to oxygen, 15 $cm^3 \cdot m^{-2} \cdot day^{-1} \cdot bar^{-1}$ at 23 ºC and 0% RH) and cooked in a cooking pot containing 50 L of tap water as follows: first, they were heated at 40 ºC for 2 h, after which the temperature was increased to 60 ºC and maintained at this temperature for 2 h; the temperature was then increased to 78 ºC until the interior of

the sausage reached a temperature of 72 ºC. The sausages were then removed from the cooking pot and allowed to cool at room temperature. Those sausages intended for chemical analyses were then stored for 0, 60 and 120 days at 4 ºC, whereas those intended for sensory analysis were stored for 60 days at 4 ºC. Following the storage period, sausages were finely ground (Robot Coupe mixer, model BX3; Jackson, MS, USA), vacuum packed in high-barrier multilayer bags (Cryovac® BB325; approximately 15 g meat/bag) and stored at -25 ºC until analysis. Unless otherwise specified, each sample was analyzed twice and the average of the obtained results was treated as a single measurement.

2.4. Moisture and pH determination

The moisture of the samples (initial raw mixtures and cooked sausages) was determined using the ISO 1442 procedure (International Organization for Standardization, 1997) and used to express some of the results on a dry-weight basis. The measurement of pH in the samples (initial raw mixtures and final raw mixtures before and after each fermentation type) was carried out in quintuplicate using a pH meter (Crison pH 25 model; Crison Instruments, S.A., Alella, Spain); the average was treated as a single measurement.

2.5. Determination of crude fat content and fatty acid composition

The fat content of the raw mixtures was measured in accordance with the AOAC Official Method 991.36 (AOAC, 2000). The fatty acid composition of raw mixtures was determined by gas chromatography (Bou et al., 2005). First, lipid extraction was carried out with 20 mL chloroform/methanol (2:1, v/v) in 1.5 g raw mixture, which was subsequently re-extracted twice using 10 mL of the same solvent mixture each time. Fatty acid methyl esters were then prepared from this fraction using sodium methoxide and BF_3. Fat content was expressed on a fresh-weight basis, whereas fatty acid composition was expressed as a percentage of area normalization.

2.6. Microbiological analysis

Twenty-five grams of either raw mixture or fermented sausage were taken aseptically and homogenized with 75 mL of buffered peptone water (BPW; OXOID, Basingstoke, UK) for 2 min in an IUL masticator (IUL S.A., Barcelona, Spain). Serial decimal dilutions were made in sterile Ringer ¼ solution (Scharlau, Barcelona, Spain). The following food-borne pathogens were determined in the raw sausages: *Escherichia coli* was enumerated on MacConkey agar (OXOID) and *Staphylococcus aureus* on Mannitol salt agar (MSA; OXOID); and the population of sulfite-reducing clostridia was determined by counting on SPS agar (Scharlau) anaerobically. All agars were incubated at 37 ºC for 48 h. Oxidase test, growth on EMB agar (OXOID), the indole test (Bell et al. 2005) and API 20E identification strips (bioMérieux) were used to identify lactose-positive colonies on MacConkey agar. DNAse and catalase production, the coagulase

test (Bell et al. 2005) and API STAPH identification strips (bioMérieux) were used to identify mannitol-positive colonies on MSA. The absence of *Salmonella* was determined by pre-enrichment in BPW for 16 h at 37 ºC, enrichment in Selenite Cystine Broth (OXOID) for 24 h at 37 ºC and in Rappaport Vassiliadis Broth (OXOID) for 24 h at 42 ºC, and isolation on SS agar (OXOID) and DCLS agar (OXOID). Both agars were incubated for 48 h at 37 ºC. Kligler Iron agar (OXOID), Lysine Iron agar (OXOID), Urease Broth (OXOID) and API 20E system (bioMérieux España) were used to identify colonies grown on SS agar and/or DCLS agar. Starter bacteria were analyzed before and after fermentation by spread plating on MRS agar (OXOID) for lactic acid bacteria and on MSA (OXOID) for staphylococci. Both cultures were incubated at 30 ºC for 3 days. Colonies from countable plates were initially tested for morphology, Gram stain, catalase production and nitrate reductase activity (Bell et al., 2005). The API STAPH system (bioMérieux) was used to identify *Micrococcaceae.* Gram stain and API 20C AUX identification strips (bioMérieux) were used to identify yeasts grown on MRS agar plates. Microorganisms are destroyed by cooking, and therefore lactobacilli and total staphylococci were not analyzed at the different storage time points.

2.7. Nitrate and nitrite determination

Determination of the residual nitrate and nitrite contents was based on Griess reaction method as described elsewhere (Magrinya et al., 2009). Nitrate and nitrite determination

was carried out in the raw mixtures after the addition of nitrite sources and in the sausages following different lengths of storage.

2.8. Total and cured pigment analysis

The mononitrosylhemochrome and total pigment concentrations of the cooked sausages were measured after extraction in 80% acetone and acidified acetone, respectively, using the Hornsey's method (Wrolstad, 2005).

2.9. Color measurements

Color was measured using a Konica Minolta Chroma-Meter (model CR-410; Konica Minolta Sensing Inc., Osaka, Japan) based on the CIE *L*a*b** color space. CIE (Commission Internationale de l'Eclairage) *L** (lightness), *a** (redness/greenness) and *b** (yellowness/blueness) were determined from five different random surfaces of the ground cooked sausages and the average of each parameter was treated as a single measurement. The instrument was set for illuminant D-65 and at a 2º observer angle and standardized using a standard white plate. The CIE *L*a*b** color space was transformed into the *L*C*h* color space, where *L** represents lightness, *C** represents chroma, and *h* represents the hue angle, as described elsewhere (Magrinya et al., 2012).

2.10. Tocopherol and tocotrienol determination

Tocopherols and tocotrienols were determined by HPLC as described elsewhere (Magrinya et al., 2012). Two grams of ground raw mixture or sausage samples were saponified at 70 ºC for 30 minutes with methanolic KOH. Then, the unsaponifiable matter was extracted with petroleum ether. The extract was filtered, evaporated and dissolved in n-hexane prior to HPLC determination. Results were expressed as mg of each tocopherol or tocotrienol per kg on a dry-weight basis.

2.11. Oxidative status and susceptibility to oxidation

The ferrous oxidation-xylenol orange (FOX) method was used to measure the lipid hydroperoxide (LHP) content and the susceptibility of the samples to oxidation after 144 hours of incubation to assess the samples' susceptibility to oxidation, as described elsewhere (Tres et al., 2009). This latter induced FOX assay to measure susceptibility to oxidation was carried out only once in the non-stored cooked sausages. Thiobarbituric acid (TBA) values were determined to assess secondary oxidation after the acid aqueous extraction of the samples through third-derivative spectrophotometry (Grau et al., 2000). LHP content and TBA values were determined in all cooked sausages.

2.12. Sensory analysis

Samples stored at 4 ºC for 60 days were randomly presented to the participants in a balanced incomplete block design (Cochran and Cox, 1957): 12 blocks, five samples per block and five replicates for each sample treatment. This design was performed in

triplicate by using 36 members from the institute to evaluate the overall acceptability of the product. The selection criteria were to consume this type of product or other cooked cured meat products and be familiarized with acceptance tests. Each panelist evaluated the acceptability of a blind control (in total six samples were given to each panelist), which was a commercial sausage. Each panelist had several slices of the samples and the blind control sausage, which were placed on white plastic dishes, identified by random three-digit numbers. Water and unsalted crackers were provided to panelists to cleanse their palates between each sample. Panelists were asked to score the overall acceptability of the product on a nine-point hedonic scale. The blind control scores were subtracted from their respective sample acceptability scores.

2.13. Statistical analyses

A multifactor ANOVA was used to identify differences produced by the different factors in terms of sample moisture, microbial counts, pH, residual nitrate and nitrite content, mononitrosylhemochrome and total pigment concentrations, color measurements, tocopherols and tocotrienols, TBA values, LHP content, susceptibility to oxidation (induced FOX assay, AUC) and overall acceptability. The factors studied were tocopherol extract addition (0 and 200 mg of tocopherols/kg), fermentation conditions for 12 h (at 16 ºC with bioprotective and nitrate-reducing cultures, at 4 ºC with nitrate-reducing culture and at 4 ºC without starter cultures), nitrite source (pure sodium nitrite or VC) and storage time (0, 60 and 120 days at 4 ºC). Microbiological

analyses, pH measurement and induced FOX assay were only conducted before storage, and overall acceptability was evaluated only after 60 days of storage. Interactions between more than two factors were ignored. When significant interactions were found between two factors, a series of one-way ANOVAs (for factors with more than two levels) or *t*-tests (for factors with two levels) were performed for each factor by fixing the other factor at each specific level. In all cases, $P \leq 0.05$ was considered significant. When significant differences were found through the multifactor or one-way ANOVAs, the least-squares means and means were separated using Scheffé's test (α=0.05).

3. RESULTS AND DISCUSSION

3.1. Moisture, crude fat, fatty acid composition, pH and tocopherol and tocotrienol content of raw mixtures

The moisture of both raw mixtures, with and without tocopherol extract, was 65.5±0.4%. The crude fat content of the raw mixture was 12.9±0.24% with tocopherol extract and 13.4±0.28% without tocopherol extract. The relative percentages of saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids of the raw mixture with the addition of the tocopherol extract were 38.29, 49.56, and 12.15%, respectively, whereas for the raw mixture without the addition of the tocopherol extract, they were 38.70, 49.26, and 12.04%, respectively. The pHs of raw mixtures with and without tocopherol extract were 5.72±0.06 and 5.72±0.07, respectively. Therefore, the

moisture, crude fat content, fatty acid composition and pH did not differ significantly between raw mixtures.

The α-tocopherol, β-tocopherol, γ-tocopherol, and α-tocotrienol content in the raw mixture without tocopherol extract averaged 10.7±0.73, 0.33±0.038, 0.31±0.031 and 0.40±0.010 mg/kg, expressed as dry weight, respectively, whereas in the raw mixture containing the tocopherol extract the α-tocopherol, β-tocopherol, γ-tocopherol, and δ-tocopherol averaged 81.5±6.31, 8.1±2.58, 209±10.6 and 31.1±2.08 mg/kg.

3.2. Microbiological analyses and pH determination

The raw mixtures were checked for the presence of food-poisoning bacteria. *E. coli* and *S. aureus* accounted for less than 4 x 10^2 cfu/g; *Salmonella* sp. was absent in 25 g; and sulfite-reducing clostridia accounted for less than 10 cfu/g. Raw mixtures met microbiological standards for raw minced meat.

Lactobacilli and total staphylococci were analyzed in raw mixtures following the addition of the starters and in sausages after the three types of fermentation. In type A, a mixture of *L. sakei*, *S. xylosus* and *S. carnosus* was added to the raw mixtures at 10^6 cfu/g, as recommended by the manufacturer, and sausages were fermented at 16 ºC for 12 h. In type B, only *S. carnosus* starter culture was added and fermentation was conducted at 4 °C for 12 h. This time and temperature conditions are currently used not only in the production of this sausage but also in the production of cooked ham. In type

C, the sausages were kept at 4 ºC for 12 h without the addition of starter cultures and thus serves as a negative control of the latter. Table 2 summarizes the results obtained from the fermented sausages. The lactobacilli and staphylococci were affected by the fermentation type used. The highest counts of lactobacilli on MRS agar were found in type A fermentation. Before fermentation the pH was 5.66 for all treatments. A clear drop in pH (from 5.66 to 5.30) occurred after 12 hours at 16 ºC using type A fermentation (Table 2). *L. sakei* produces lactic acid and increases acidification during fermentation. Therefore, the pH drop was due to the lactic acid produced by the starter culture used.

There were no significant differences between the concentration of total staphylococci before and after the fermentation process (data not shown). Thus the sausages fermented at 4 ºC (type B) had the same cfu/g as those fermented at 16 ºC (type A), suggesting that mild temperatures are not sufficient to promote the growth of these bacteria. However, staphylococcal strains belonging to *S. xylosus* and *S. carnosus* were reported to reduce nitrate to nitrite at 15 ºC, 20 ºC and 30 ºC (Casaburi et al., 2005; Mauriello et al., 2004; Miralles et al., 1996). Thus, the residual nitrite concentration after fermentation was higher in sausages fermented with *S. carnosus* at 4 ºC (type B) than in those fermented with two nitrate-reductase active staphylococci (*S. carnosus* and *S. xylosus*) at 16 ºC (type A) (Table 3).

In the type C sausages, the initial counts on the MRS were estimated at 5.9 log cfu/g. The overgrowth of yeasts on the MRS plates and the fact that no LAB were retrieved from any of the MRS plates led us to estimate LAB counts at less than 2.6 log cfu/g, which was the detection limit of the agar plate method. The yeast species found in our samples and identified as *Candida zeylanoides*, *C. lipolytica* and *C. famata*, are considered psychrotrophic. *C. parapsilosis* was also detected in the sausage mixture. The presence of these yeast species has been reported in salami, fresh sausages and Spanish fermented sausages (Encinas et al., 2000; Gardini et al., 2001). The initial content of staphylococci was 4.7 log cfu/g; 12 of the isolates belonged to *S. xylosus* and three isolates were identified as *S. sciuri* or *S. capitis*. Both, *S. sciuri* and *S. capitis* have been isolated from dried fermented sausage (Papamanoli et al., 2002). Nitrate reductase activity was observed for ten isolated *S. xylosus* strains. Our results are similar to those reported by Mauriello et al. (2004) and Casaburi et al. (2005). Total counts on the MRS agar plates increased by less than one log cfu/g after 12 h of fermentation. The cell number increase on the MRS was due to yeasts, and the final number of lactobacilli was 2.6 log cfu/g (Table 2). The number of total staphylococci found after fermentation is in line with that reported by Miralles et al. (1996) in naturally fermented sausages produced without the addition of starter cultures. The yeasts and nitrate-reductase–active staphylococci found in these sausages may affect product quality, although to a much lesser extent than starter cultures (Mauriello et al., 2004).

Sausages in which pure sodium nitrite was added contained significantly more lactobacilli than sausages made with the VC (Table 2). Plants, herbs and spices have been reported as sources of natural antimicrobials, and therefore the lower concentration of lactobacilli could be due to the antimicrobial properties of the VC, which is made from celery and carrot (Palou et al., 2005).

3.3. Nitrate and nitrite residual amounts

The residual nitrate and nitrite levels in sausages are presented in Table 3. In all cases, sausages fermented with *S. carnosus* were far below the limit established for organic production in Europe (European Commission, 2008).

Significant differences were observed for residual nitrate and nitrite content depending on fermentation type, nitrite source and storage time. Type C fermentation and the use of VC as a curing agent led to higher residual nitrate levels (Table 3). There was a significant interaction between the nitrite source and fermentation type for the residual nitrate amount (Figure 1). As expected, sausages without starter cultures (type C) had higher amounts of residual nitrate, especially in those sausages formulated with the nitrate-rich VC. Although it was found that there is microbiota with nitrate reductase activity in type C sausages, when nitrate-rich VC is used, a nitrate-reducing bacterial culture is required for the curing process (Sebranek and Bacus, 2007; Sindelar et al., 2007b).

Sausages produced with type B fermentation and those produced with pure sodium nitrite contained higher amounts of residual nitrite (Table 3). This is because the interaction between nitrite source and fermentation type significantly influenced residual nitrite levels (Figure 1). Sausages formulated with VC and subjected to type C fermentation contained much lower residual nitrite amounts than the corresponding sausages formulated with pure $NaNO_2$. This is because the reduction reaction from nitrate to nitrite was very low when fermentation was conducted at 4 ºC for 12 h without starter cultures, and is consistent with the high amounts of residual nitrate found in those sausages formulated with VC and fermented in these conditions (Figure 1). However, when pure nitrite is added the nitrate and nitrite residual levels were alike for type B and type C fermentations. On the other hand, the amounts of residual nitrite found in type A and type B sausages, fermented with a nitrate-reducing starter culture (*S. carnosus*), did not differ significantly between nitrite sources (Figure 1).

The depletion of nitrite during the storage of cooked cured meat products is a widely recognized phenomenon (Sindelar et al., 2007a; Sindelar et al., 2007b; Krause et al., 2011; Terns et al., 2011a; Terns et al., 2011b), and was also observed in this study (Table 3). However, residual nitrate levels showed a different pattern of behavior during storage (Table 3). The regeneration of nitrate from nitrite is not uncommon in meats (Magrinya et al., 2012; Sindelar et al., 2010; Terns et al., 2011a; Terns et al., 2011b; Tsoukalas et al., 2011), and was attributed to an oxidative reaction between the added

nitrite and various compounds present in the food matrix. The conversion to nitrate upon storage is confirmed to occur in this study in which even lower ingoing amounts of nitrite sources were assessed (equivalent to 70 instead of 80 mg $NaNO_2$/kg). Despite this increase in residual nitrate over time, the sum of the residual nitrate and nitrite levels, expressed as nitrate ion, decreased with storage time, which indicates that the curing agents could be involved in curing reactions during the storage of the cooked sausages.

3.4. Total and cured pigment analyses

The mononitrosylhemochrome concentration and curing efficiency of the sausages is presented in Table 3. Cured meat products are considered acceptable when the pigment conversion ratio is 80% or higher (Wrolstad, 2005). The main effects of fermentation type and nitrite source were significant for nitrosylhemochrome concentration and curing efficiency (Table 3). The lowest concentration of the characteristic cured pigment was found in type C sausages to which no starter cultures were added. The curing efficiency for sausages subjected to type A and B fermentations was above 80% thus meaning that an optimum curing can be achieved with relatively low amounts of nitrite (70 mg $NaNO_2$/kg). These results are in line with previous results at nitrite sources concentrations equivalent to 80 mg $NaNO_2$/kg (Magrinya et al., 2012).

Table 3 shows that VC affected the curing process, because those sausages in which this nitrite source was used seemed to have a lower curing efficiency. However, these misleading results can be explained by the interaction between the fermentation type and nitrite source factors (Figure 2). Those sausages formulated with the VC in combination with type C fermentation showed much lower concentrations of nitrosylhemochrome and a much lower curing efficiency, but no differences were found between the three fermentation conditions when pure sodium nitrite was used (Figure 2). Therefore, with type C fermentation only it is possible to produce organic *botifarra catalana* with an appropriate curing efficiency with the addition of pure nitrite at 70 mg/kg. As expected, when the sausages were formulated with a nitrate-rich VC as a source of nitrite, the lack of a starter culture with nitrate reductase activity resulted in sausages with much lower levels of cured pigment, which demonstrates the crucial role of *S. carnosus* during fermentation in producing cooked cured sausages when this source of nitrite is used (Casaburi et al., 2005). These results suggest that when pure nitrite is added it may be possible to produce sausages with an optimal curing by cooking them immediately. That means the omission of the storage at 4 °C and, in consequence, the elaboration of *botifarra catalana* under faster processing conditions than those of type A. In case that a nitrate source is added, then it is necessary to use *S. carnosus* but the effects of the omission of storage at 4 °C and, therefore, the direct submission of sausages to a progressive cooking should be assessed. However, it should

be borne in mind that these faster production processes may affect to the aroma and flavor development of this product typically fermented at low temperatures and, in consequence, the acceptability of these products should be considered.

3.5. Color measurements

The fermentation type and nitrite source factors influenced all of the studied instrumental color parameters (Table 3). Lightness (L^*) was higher in type A sausages fermented at 16 ºC for 12 h with the bioprotective starter culture (*L. sakei* and *S. xylosus*) and the nitrate-reducing culture (*S. carnosus*). The color and texture of the sausages depended on protein denaturation caused by the decline in pH and heat treatments. With respect to the decline in pH, it has been reported that salami fermented with lactic acid bacteria resulted in higher L^* and a^* values (Barbut, 2010). The author also found that cooked sausages had higher L^* values than raw meat mixtures. Therefore, it is reasonable that those sausages initially fermented at 16 ºC for 12 h having lower pH values (Table 2) resulted in higher L^* after cooking when compared with other sausages in which there was no obvious decline in pH. Increased redness was found in those sausages fermented with *S. carnosus*, regardless of fermentation temperature. The a^* value is related to visible redness in meat and the content of nitrosylhemochrome (Barbut, 2010). Therefore, the lower a^* value in sausages without

starter cultures (type C) could be explained by the lack of nitrate-to-nitrite conversion and the inhibition of the subsequent curing process.

According to the results shown in Table 3, the addition of pure sodium nitrite to the sausage formula as a curing agent produced darker and redder sausages with higher color saturation. This is consistent with Krause et al. (2011), who found that hams formulated with pure sodium nitrite had a more intense cured color than those cured with vegetable juice powder. In addition, a significant interaction between the fermentation type and nitrite source was found for the color parameters (Figure 3). Therefore, as mentioned above, when sausages were formulated with VC, nitrate was efficiently reduced to nitrite by the nitrate-reducing starter culture and consequently the cured pigment was efficiently formed in these sausages (Figures 1 and 2). As a result, these sausages had the same a^* values as those sausages produced using pure sodium nitrite (Figure 3). Terns et al. (2011b) reported similar results in cooked cured sausages. The interactions found for the hue angle and chroma values are probably also a consequence of the intense nitrate reductase activity of the *S. carnosus* culture. In addition, several authors (Terns et al., 2011a; Terns et al., 2011b; Tsoukalas et al., 2011; Magrinya et al., 2012) have found that similar VC produced lighter and yellower sausages compared to those made with pure sodium nitrite, and this was also attributed to the intrinsic color of the powder.

The color of the sausages was constant throughout storage. Likewise, lightness and yellowness have been reported to show no consistent variations in vacuum-packed bologna sausages (Carballo et al., 1991) and sliced ham (Krause et al., 2011) stored for 42 or more days in the dark under refrigeration. However, the decrease in *a** values during storage has been reported in vaccum-packed cured sausages (Terns et al., 2011b) and also in *botifarra catalana* (Magrinya et al., 2012) that was associated with degradation of the cured pigment. In other studies *a** values increased during storage and this was explained by the fact that residual nitrite reacted with myoglobin during storage and produced colored pigments (Terns et al., 2011a; Sindelar et al., 2007b). The fact that the amount of cured sausage pigment in the present study was constant during storage (Table 3) could explain the color stability of *botifarra catalana* during vacuum-packed refrigerated storage. The decrease in the sum of residual nitrate and nitrite amounts observed during storage of the cooked sausages in this study seems to be in agreement with their role as a reservoir to maintain red color.

3.6. Tocopherol and tocotrienol content

The tocopherol content of the sausages is reported in Table 4. The addition of the tocopherol extract to the formula led to significant changes in amounts of the different tocopherols in the sausages. The extract is particularly rich in γ-tocopherol (Table 1 footnote), which explains the high content of this tocopherol in sausages and the raw mixture containing this tocopherol extract. It is interesting to note that the cooking

procedure had no significant effect on the tocopherol content. Therefore, it is possible to add a tocopherol extract to raw meat mixtures to produce cooked cured sausages enriched with tocopherols. The interactions between the other studied factors had no significant effects on the tocopherol and tocotrienol content of the sausages.

3.7. Oxidative status and susceptibility to oxidation

The LHP content of the sausages is shown in Table 4. In comparison with other studies (Magrinya et al., 2012; Magrinya et al., 2009), the LHP content was low, but significant enough to show differences based on fermentation type and storage time. Type C sausages contained the highest amounts of LHP. It is well known that nitrite acts as an antioxidant in cured meats (Pegg and Shahidi, 2000). Therefore, the absence of nitrate-reducing starter cultures in samples containing nitrate, provided by means of the VC, decreased the formation of nitrite thus explaining the higher LHP content.

With respect to secondary oxidation, no significant differences were found in the TBA values of sausages for the main factors studied (Table 4). TBA values were higher than those found in a previous study (Magrinya et al., 2012) which can be attributed to a number of reasons including the lower ingoing nitrite levels. Despite that, the recorded values were consistent with other studies dealing with other cooked cured meat products (Parra et al., 2010).

In dry-fermented sausages, the addition of tocopherol extract was found to prevent oxidation (Magrinya et al., 2009). However, in the present study, it had no significant effect during the production and storage of *botifarra catalana*. This difference is probably due to the short time elapsed between the addition of the tocopherol extract and the vacuum packaging of the *catalana* sausages in low permeability plastic bags. Various authors have reported that hams manufactured from pigs whose diet was supplemented with α-tocopheryl acetate exhibited a higher degree of oxidative stability during retail storage (DeWinne and Dirinck, 1997; Dineen et al., 2000). This could be also related to the fact that dietary supplementation with tocopheryl acetate has already been shown to be more effective against oxidation than the *post mortem* addition of tocopherol to meat (Jensen et al., 1998).

TBA values did not increase significantly during storage, as other authors have also found for vacuum-packed cured meat products (Carballo et al., 1991; Dineen et al., 2000; Parra et al., 2010). One explanation could be the higher oxidative stability of vacuum-packed cured products. Furthermore, sodium nitrite has been shown to be an effective antioxidant at levels as low as 50 mg/kg of ingoing nitrite (Pegg and Shahidi, 2000).

3.8. Sensory analysis

The results for the overall acceptability test carried out after 60 days storage at 4 ± 2 ºC in vacuum-packed sealed bags are shown in Table 4. There were no differences in the overall acceptability of the sausages containing the tocopherol extract compared with control sausages (Table 4). In hams, De Winne and Dirinck (1997) found differences between the control and those with a higher content of α-tocopherol using a triangle test and a paired comparison. The results indicated that the supplemented ham had a fresher odor and taste and these attributes were related to lipid oxidation. TBARS values of 0.5 to 1.0 mg/kg have been suggested as the threshold for oxidized odor and 1.0 to 2.0 mg/kg for oxidized flavor (Tarladgis et al., 1960). Therefore, the fact that all TBA values were within the oxidized odor level and did not differ between treatments (Table 4) could explain why panelists found no differences between sausages with and without the tocopherol extract.

Meat-purchasing decisions are influenced by color more than any other quality factor since consumers use discoloration as an indicator of freshness and wholesomeness. This may explain why those sausages with the lowest scores presented less redness and chroma and a higher hue angle (Table 3). In this respect, the lack of nitrate-reducing culture is crucial for color development upon the addition of VC and thus negatively affects overall acceptability.

Therefore, the use of nitrate reductase cultures not only helps develop a cured color but may also influence on overall acceptability. In fact, in a previous study, panelists

preferred *botifarra catalana* made with the same VC at 0.33%, even though sausages made with pure sodium nitrite were slightly redder (Magrinya et al., 2012). In hams, trained panelists indicated that a vegetable aroma from VC can be detected when this was added at concentrations about 0.3% (Sindelar et al., 2007a). Moreover, in comparison with the addition of pure sodium nitrite, consumers showed no preference in terms of overall acceptability for emulsified cooked sausages with added vegetable juice powder at 0.2% (Terns et al., 2011b). Thus, it is not clear whether consumers could detect the presence of VC and whether they had a preference for these sausages, but no dislike was expressed as long as the color was sufficiently red.

To conclude, it is possible to manufacture organic cooked cured sausages without the addition of pure nitrite. In case of omission of pure nitrite, nitrate-rich VC can be used as curing agents in fermented meat products. However, residual nitrate and nitrite should be minimized to avoid nitrosamine formation. The use of nitrate-reducing bacteria is an interesting approach to reducing these residual amounts and controlling curing reactions, even when the only curing agent used is nitrite. In the presence of nitrate, the addition of nitrate-reducing cultures caused the appropriate curing of the meat product and helped to decrease the formation of hydroperoxides. Conversely, the addition of tocopherols in our conditions was found to have no effect on oxidative status

and acceptability scores. Therefore, it is advisable to produce organic *botifarra catalana* using VC in combination with cultures with intense nitrate reductase activity.

FIGURE CAPTIONS:

Fig. 1 Interaction between fermentation type and nitrite source for the residual nitrate and nitrite content in cooked sausages

Fig. 2 Interaction between fermentation type and nitrite source for nitrosylhemochrome concentration and curing efficiency in cooked sausages

Fig. 3 Interaction between fermentation type and nitrite source for lightness (L*), redness (a*), chroma (C*) and hue angle (h) in cooked sausages

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Table 1. Sausage treatments

Tocopherols (mg/kg)[a]	Fermentation [b]	Nitrite source [c]
200	Type A	Pure sodium nitrite
200	Type A	Vegetable concentrate
200	Type B	Pure sodium nitrite
200	Type B	Vegetable concentrate
200	Type C	Pure sodium nitrite
200	Type C	Vegetable concentrate
0	Type A	Pure sodium nitrite
0	Type A	Vegetable concentrate
0	Type B	Pure sodium nitrite
0	Type B	Vegetable concentrate
0	Type C	Pure sodium nitrite
0	Type C	Vegetable concentrate

[a] Expressed as average sum of tocopherols in mg/kg of raw mixture. The tocopherol extract contained α-, β-, γ-, and δ-tocopherols at the concentrations of 82±2, 8.2±0.3, 293±9, and 110±2 g/kg, respectively.

[b] Three different fermentation types, where A involved 12 h at 16 ºC and contained a bioprotective starter culture, *Lactobacillus sakei* and *Staphylococcus xylosus*, and a nitrate-reducing culture, *Staphylococcus carnosus*; B involved 12 h at 4 ºC and contained a nitrate-reducing culture, *Staphylococcus carnosus*; and C involved 12 h at 4 ºC and no starter cultures.

[c] Addition of pure $NaNO_2$ or vegetable concentrate, each providing the equivalent of 70 mg $NaNO_2$/kg raw mixture.

Table 2. Effect of addition of tocopherol extract, fermentation type and nitrite source on microbial counts and pH of fermented *botifarra catalana* before cooking.[a]

	Lactobacilli (log cfu/g)[b]	Staphylococci (log cfu/g)[c]	pH after fermentation
Tocopherol (mg/kg)			
0	5.38	5.94	5.52
200	5.45	5.76	5.55
SEM	0.021	0.108	0.010
Fermentation			
Type A	7.68 z	6.30 y	5.30 x
Type B	5.95 y	6.35 y	5.62 y
Type C	2.60 x	4.90 x	5.68 y
SEM	0.026	0.132	0.013
Nitrite source			
Pure sodium nitrite	5.55 y	5.89	5.51
Vegetable concentrate	5.28 x	5.81	5.55
SEM	0.210	0.108	0.010

[a] The description of the different effects is provided in Table 1. Values given in this table correspond to least-squares means obtained from multifactor ANOVA (n = 12 for lactobacilli, staphylococci, and pH). Least-squares means within the same column for the same factor but with different letters differ significantly ($P \leq 0.05$).

[b] Microbial counts expressed as the logarithm of lactobacilli colony-forming units per g of dried sample.

[c] Microbial counts expressed as the logarithm of staphylococci colony-forming units per g of dried sample.

Table 3. Effect of addition of tocopherol extract, fermentation type, nitrite source, and storage time on residual nitrate and nitrite, mononitrosylhemochrome, curing efficiency and instrumental color of cooked cured *botifarra catalana*[a].

	Residual nitrate[b] (mg/kg)	Residual nitrite[c] (mg/kg)	Mononitrosyl-hemochrome[d] (mg/kg)	Curing efficiency[e] (%)	Instrumental color[f]			
					*L**	*a**	C*	*h*
Tocopherol								
0	10.3 x	2.3	179	77.8	63.47	15.73	17.92	28.70
200	11.2 y	2.5	174	77.4	63.51	15.64	17.84	28.81
SEM	0.32	0.40	2.0	0.56	0.094	0.058	0.042	0.156
Fermentation								
Type A	1.7 x	0.4 x	198 y	86.2 z	63.73 y	16.31 y	18.36 y	27.31 x
Type B	2.5 x	4.5 y	189 y	83.2 y	63.23 x	16.34 y	18.37 y	27.18 x
Type C	28.0 y	2.3 x	142 x	63.3 x	63.47 xy	14.04 x	16.92 x	31.78 y
SEM	0.39	0.49	2.5	0.69	0.115	0.071	0.051	0.192

Nitrite source								
Pure sodium nitrite	5.0 x	3.2 y	191 y	83.6 y	63.24 x	16.47 y	18.44y	26.71 x
Vegetable concentrate	16.6 y	1.7 x	162 x	71.5 x	63.74 y	14.90 x	17.33 x	30.79 y
SEM	0.32	0.40	2.0	0.56	0.094	0.058	0.042	0.156
Storage time (days)								
0	9.9 x	5.4 y	177	78.0	63.26	15.74	17.91	28.60
60	11.5 y	1.3 x	173	76.7	63.54	15.68	17.89	28.83
120	10.6 xy	0.5 x	180	78.0	63.68	15.64	17.85	28.83
SEM	0.39	0.49	2.5	0.69	0.115	0.071	0.051	0.192

[a] The description of the different effects is provided in Table 1. Values given in this table correspond to least-squares means obtained from multifactor ANOVA (each determination has a n = 36). Least-squares means within the same column for the same factor but with different letters differ significantly ($P \leq 0.05$).

[b] Residual nitrate is expressed as mg of $NaNO_3$ per kg of sausage.

[c] Residual nitrite is expressed as mg of $NaNO_2$ per kg of sausage.

[d] Results are expressed as mg mononitrosylhemochrome per kg of sausage as dry weight.

[e] Curing efficiency expressed as the percentage of the concentration of mononitrosylhemochrome divided by the concentration of total heme pigments, both concentrations expressed per kg of sausage as dry weight.

[f] L*, lightness; a*, redness; b*, yellowness; chroma (C*) is the root of the sum of the squares of a* and b* and is used to express color saturation; hue angle (h) is the arctangent of the quotient of b*/a* and is used to express color hue (h = 0, true red; h = 90, true yellow).

Table 4. Effect of addition of tocopherol extract, fermentation type, nitrite source, and storage time on tocopherols, lipid hydroperoxide (LHP) content, thiobarbituric acid (TBA) values, susceptibility to oxidation (AUC) and consumers' overall acceptability of cooked cured *botifarra catalana*[a].

	Tocopherol (mg/kg)[b]				LHP (µmol CHP eq/kg)[c]	TBA(µg MDA/kg)[d]	AUC[e] (mmol CHP eq kg^{-1} h)	Overall acceptability[f]
	α	β	γ	δ				
Tocopherol (mg/kg)								
0	10.3x	0.3x	0.4x	ND[g]	18	669	30	-0.9
200	80.1y	12.7y	210.7y	28.5	19	558	30	-0.9
SE	0.72	0.13	1.67	0.26	1.3	3.4	6.3	0.31
Fermentation								
Type A	46.0	6.5	104.7	13.8	14 x	623	27	-1.1xy
Type B	44.7	6.5	106.0	14.4	15x	579	30	0.0y
Type C	45.0	6.5	106.0	14.4	26y	640	32	-1.6x

SEM	0.88	0.16	2.05	0.32	1.6	53.1	7.7	0.37
Nitrite source								
Pure sodium nitrite	45.2	6.3	105.9	14.1	16.9	589	29.6	-0.4y
Vegetable concentrate	45.2	6.7	105.2	14.4	20.6	638	30.0	-1.5x
SEM	0.72	0.13	1.67	0.26	1.32	43.4	6.30	0.31
Storage time (days)								
0	45.1	6.5	107.1	14.5	11.7x	570		
60	45.0	6.5	104.7	14.1	18.4y	613		
120	45.6	6.5	104.9	14.1	26.1z	659		
SEM	0.88	0.16	2.05	0.32	1.61	53.1		

[a] The description of the different effects is provided in Table 1. Values given in this table correspond to least-squares means obtained from multifactor ANOVA (n = 36, 36, 36, 12, and 180 for tocopherol and tocotrienol analogs, LHP, TBA values, AUC and overall acceptability, respectively). Least-squares means within the same column for the same factor but with different letters differ significantly ($P \leq 0.05$).

[b] Results are expressed as mg of each tocopherol per kg of sausage as dry weight.

[c] Results are expressed as μmol of cumene hydroperoxide equivalents per kg of sausage as dry weight.

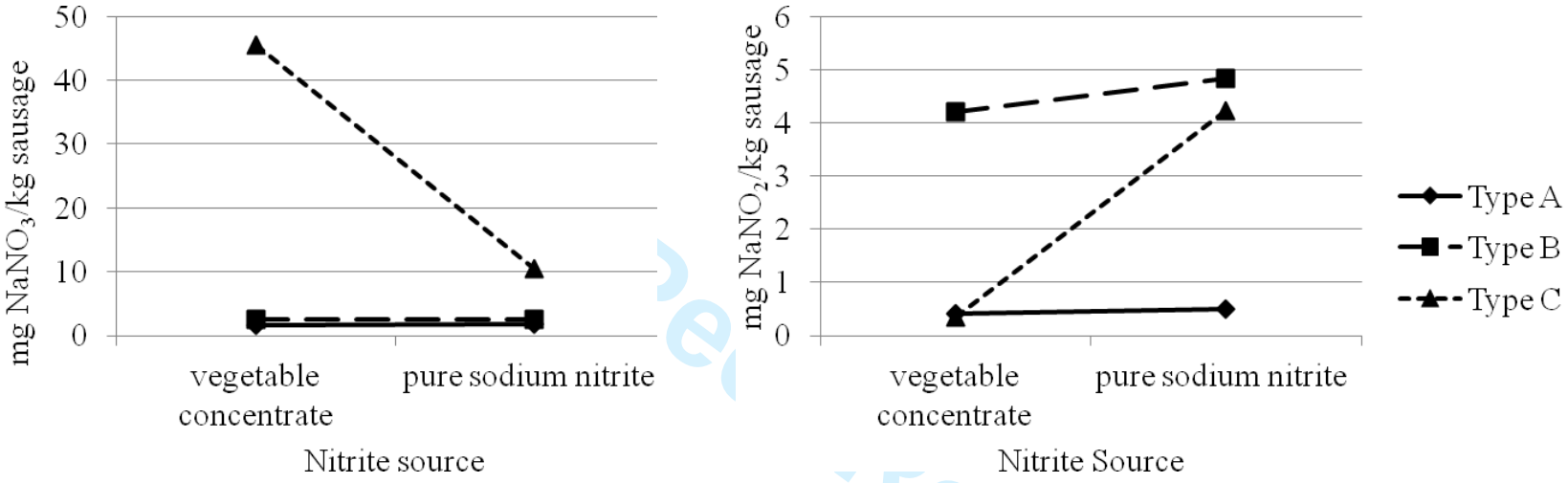
[d] Results are expressed as μg of malondialdehyde per kg of sausage as dry weight.

[e] Results are the area under the curve (AUC) of lipid hydroperoxide formation determined by means of the induced ferrous oxidation-xylenol orange (FOX) assay (incubation for 144 hours) and expressed as mmol of cumene hydroperoxide equivalents per kg of sausage as dry weight x hours. Only determined in freshly produced samples.

[f] The results for acceptability are the difference between the scores for the experimental samples and the score for a commercial blind control. Only determined after storage for 60 days.

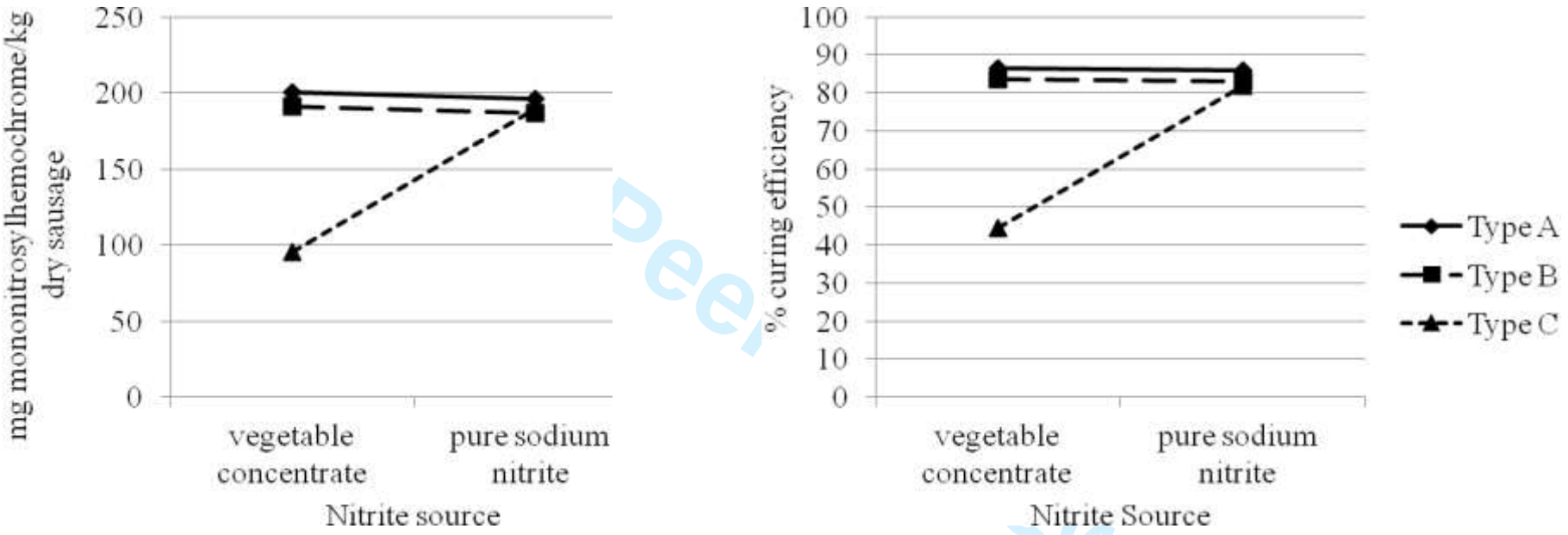
[g] ND, not detected.

Figure 1.



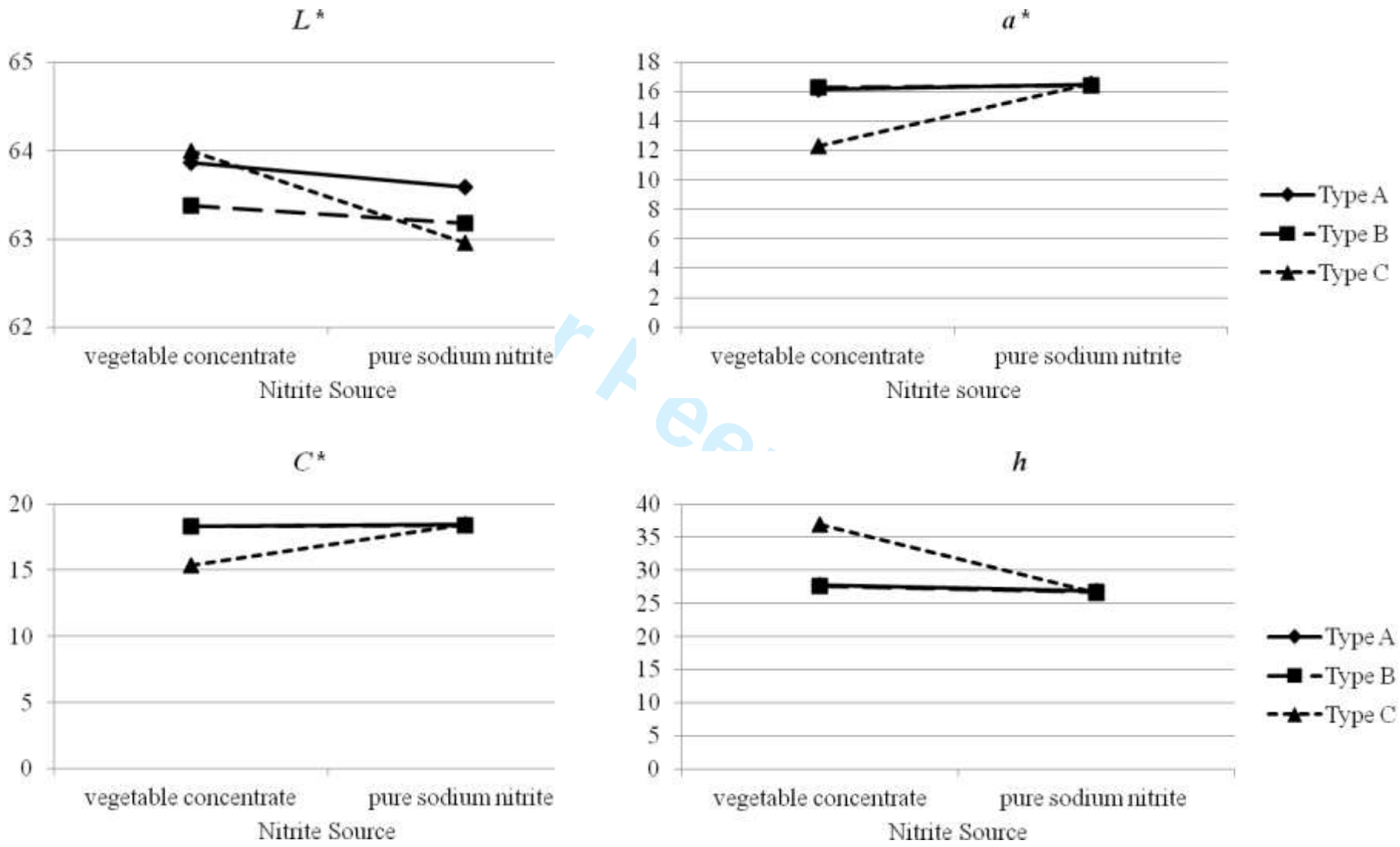
See Table 1 for description of fermentation types.

Figure 2.



See Table 1 for description of fermentation types.

Figure 3.



See Table 1 for description of fermentation types.